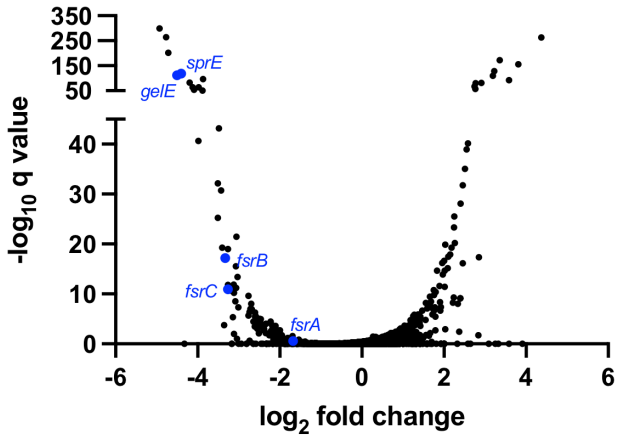
A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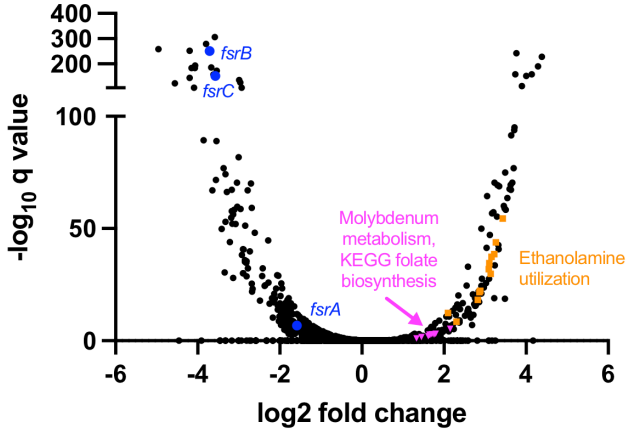
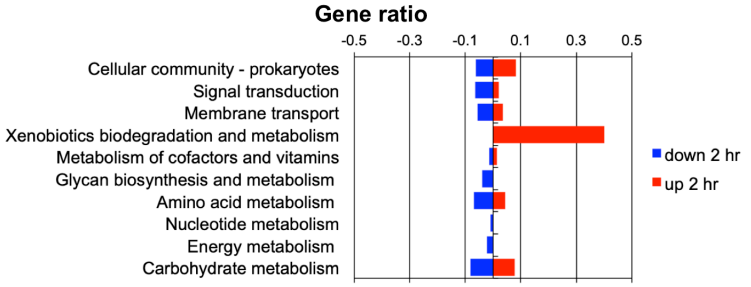
250
150
50
40
30
20
10
0
-log10 q value
sprE
gelE
fsrB
fsrC
fsrA
-6
-4
-2
0
2
4
6
log2 fold change

B


400
300
200
100
50
0
-log10 q value
fsrB
fsrC
fsrA
Molybdenum metabolism, KEGG folate biosynthesis
Ethanolamine utilization
-6
-4
-2
0
2
4
6
log2 fold change

C


Gene ratio
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-0.3
-0.1
0.1
0.3
0.5
Cellular community - prokaryotes
Signal transduction
Membrane transport
Xenobiotics biodegradation and metabolism
Metabolism of cofactors and vitamins
Glycan biosynthesis and metabolism
Amino acid metabolism
Nucleotide metabolism
Energy metabolism
Carbohydrate metabolism
down 2 hr
up 2 hr

D

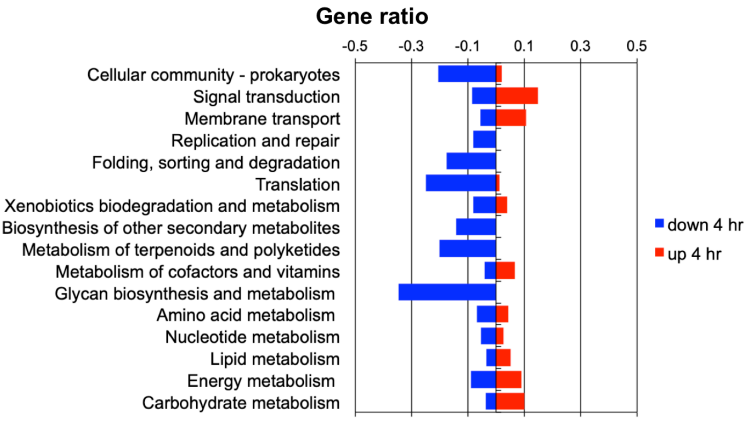
Gene ratio
-0.5
-0.3
-0.1
0.1
0.3
0.5
Cellular community - prokaryotes
Signal transduction
Membrane transport
Replication and repair
Folding, sorting and degradation
Translation
Xenobiotics biodegradation and metabolism
Biosynthesis of other secondary metabolites
Metabolism of terpenoids and polyketides
Metabolism of cofactors and vitamins
Glycan biosynthesis and metabolism
Amino acid metabolism
Nucleotide metabolism
Lipid metabolism
Energy metabolism
Carbohydrate metabolism
down 4 hr
up 4 hr

**Figure S1. Categories of differentially expressed genes in Δ*bph* after 2 and 4 h planktonic growth. A, B)** Volcano plots showing the distribution of differentially expressed genes at 2 hr (**A**) and 4 hr (**B**). **C, D)** OG1RF locus tags were converted to KEGG identifiers, and category analysis was done using KEGG Mapper for differentially expressed genes at 2 hr (**C**) and 4 hr (**D**). The graphs represent the ratio of differentially expressed genes out of the total number of OG1RF genes within each KEGG category.

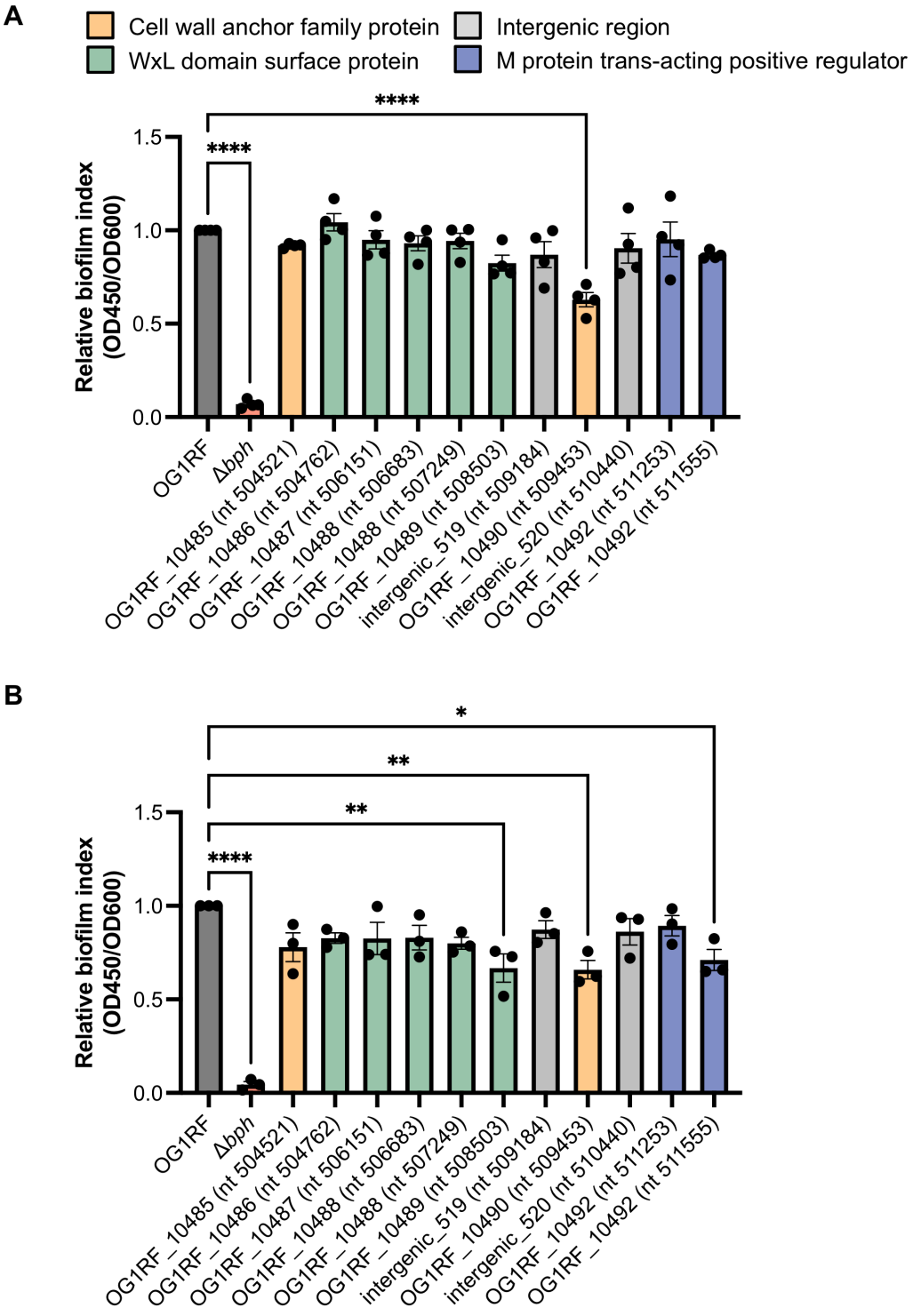


**Figure S2: Biofilm formation of mutants with Tn insertions in OG1RF_10485-10492.** Tn mutants were obtained from stock Tn library plates, and biofilm formation in TSB-D was tested in 96-well plates at **A)** 6 h and **B)** 24 h. Biofilm biomass was detected by safranin staining

($A_{450}$), and biofilm production was calculated relative to overall growth ($A_{600}$). Values were normalized to OG1RF. Each data point represents an independent biological replicate (**A**, n = 4; **B**, n = 3). Error bars represent standard error of the mean. Statistical significance was calculated by one-way ANOVA (*p<0.05, **p<0.01, ****p<0.0001).

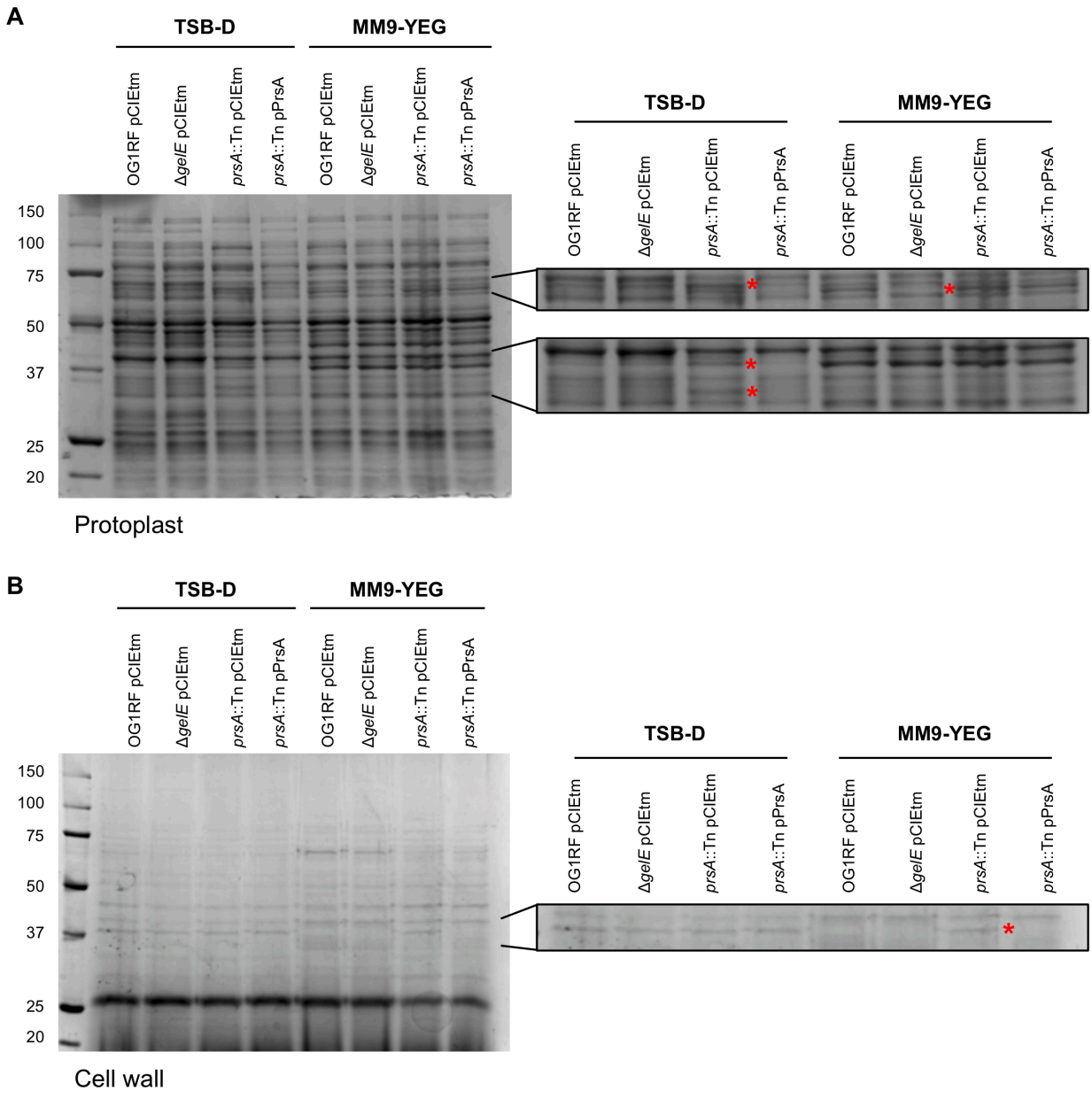


**Figure S3. Protein expression in OG1RF, Δ*gelE*, and *prsA*::Tn.** Protein lysates were prepared from **A)** protoplasts and **B**) cell wall samples. Samples were run on SDS-PAGE gels and visualized via Coomassie staining. Protein bands differentially expressed in mutant strains are marked with a red asterisk to the right of the band. Images are representative of three biological replicates.

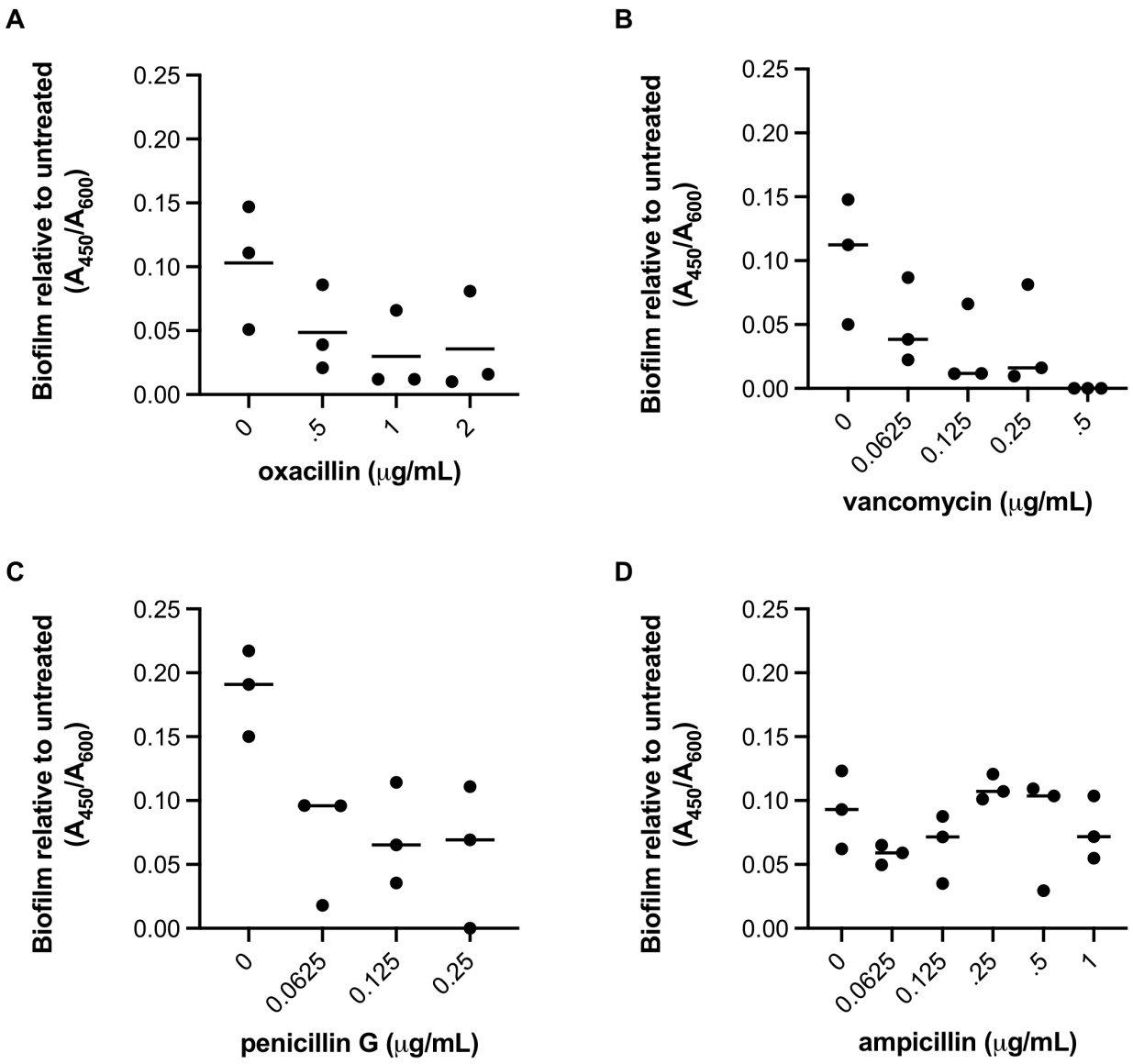


**Figure S4. Biofilm production of Δ*bph* in the presence of sub-inhibitory concentrations of antibiotics.** Δ*bph* was grown in a 2-fold dilution series of **A)** oxacillin, **B)** vancomycin, **C)** penicillin G, and **D)** ampicillin, and growth was measured as $A_{600}$. Biofilm material was stained with safranin and quantified at $A_{450}$. Biofilm production was calculated relative to OG1RF for each sample. Data points represent independent biological replicates (n = 3), and error bars show standard error of the mean.

**Table S1: RNAseq analysis of genes differentially expressed in Δ*bph* compared to OG1RF at 2 and 4 hrs in planktonic culture.**

**Table S2. Comparison of differentially expressed genes in Δ*bph* and Δ*fsrB* deletion mutants.**

**Table S3. Strains, plasmids, and oligonucleotides used in this study.**

| Strain | Description | Reference |
|---|---|---|
| *Escherichia coli* DH5α | Laboratory K-12 cloning strain | Fisher Scientific |
| *Enterococcus faecalis* OG1RF | Parent strain, $Rif^R$ $Fus^R$ | (1) |
| *E. faecalis* OG1RF Δ*bph* (JW271) | Markerless in-frame deletion of *bph* (OG1RF_10435), $Rif^R$, $Fus^R$ | (2) |
| *E. faecalis* OG1RF EfaMarTn *prsA*::Tn (JW534) | *mariner* transposon insertion at nucleotide position 440158 (OG1RF_10423), $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF Δ*gelE* (TX5264) | Markerless in-frame deletion of *gelE*, $Rif^R$, $Fus^R$ | (4) |
| *E. faecalis* OG1RF Δ*fsrA* (JD100) | Markerless in-frame deletion of *fsrA*, $Rif^R$, $Fus^R$ | (4) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10485::Tn | *mariner* transposon insertion at nucleotide position 504521, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10486::Tn | *mariner* transposon insertion at nucleotide position 504762, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10487::Tn | *mariner* transposon insertion at nucleotide position 506151, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10488::Tn | *mariner* transposon insertion at nucleotide position 506683, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10488::Tn | *mariner* transposon insertion at nucleotide position 507249, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |

| | | |
|---|---|---|
| *E. faecalis* OG1RF EfaMarTn OG1RF_10489::Tn | *mariner* transposon insertion at nucleotide position 508503, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn intergenic_519::Tn | *mariner* transposon insertion at nucleotide position 509184, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10490::Tn | *mariner* transposon insertion at nucleotide position 509453, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn intergenic_520::Tn | *mariner* transposon insertion at nucleotide position 510440, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10492::Tn | *mariner* transposon insertion at nucleotide position 511253, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| *E. faecalis* OG1RF EfaMarTn OG1RF_10492::Tn | *mariner* transposon insertion at nucleotide position 511555, $Rif^R$ $Fus^R$ $Cm^R$ | (3) |
| | | |
| **Oligonucleotide** | **Sequence and Description** | **Reference** |
| 10423-bam-fwd | 5’ – ATA GGA TCC AAA CAG GAG TGC ATA AGA G – 3’, forward primer for confirming OG1RF_10423 (*prsA*) Tn insertion and cloning into pCIEtmf, BamHI site underlined | (5) |
| 10423-nhe-rev | 5’ - TAT GCT AGC AAG GGA GTG GTC AAT CG – 3’, reverse primer for confirming OG1RF_10423 (*prsA*) Tn insertion and cloning into pCIEtm, NheI site underlined | (5) |
| fsrA-Bam-fwd | 5' - ATA GGA TCC TAA TTT TAT ATC TTC TTG AGA AAG GG - 3', forward primer for cloning *fsrA* into pCIEtm, BamHI site underlined | This study |
| fsrA-Nhe-rev | 5' - TAT GCT AGC TCC CTA AGT AAG AAA TAG CGC - 3', reverse primer for cloning *fsrA* into pCIEtm, NheI site underlined | This study |
| JD460s (16S forward) | 5’ – GCG GCT CTC TGG TCT GTA AC – 3’, forward primer to confirm depletion of genomic DNA from RNA (116 bp when paired with JD461as) | This study |
| JD461as (16S reverse) | 5’ – CGG AAA CCC TCC AAC ACT TA – 3’, reverse primer to confirm depletion of genomic | This study |

| | | |
|---|---|---|
| | DNA from RNA (116 bp when paired with JD460s) | |
| JD464s (*relA* qPCR) | 5’ – CGA CTT GCT TCG TCA GTT CA – 3’, forward primer for qRT-PCR of *relA* (106 bp when paired with JD465as) | (6) |
| JD465as (*relA* qPCR) | 5’ – ACC CAT GAG ATG CAC CAA A – 3’, reverse primer for qRT-PCR of *relA* (106 bp when paired with JD464s) | (6) |
| gelE-qPCR-fwd | 5’ – CTT TTT GGG ATG GAA AAG CA – 3’, forward primer for qRT-PCR of *gelE* (124 bp when paired with gelE-qPCR-rev) | This study |
| gelE-qPCR-rev | 5’ – CCG GCA GTA TGT TCC GTC AC – 3’, reverse primer for qRT-PCR of *gelE* (124 bp when paired with gelE-qPCR-fwd) | This study |
| | | |
| **Plasmid** | **Description** | **Reference** |
| pCIE-tet-MCS (pCIEtm) | pCIE-based plasmid vector with cCF10-inducible promoter, $Tet^R$ | (2) |
| pCIEtm::*fsrA* | pCIEtm expressing *fsrA* from cCF10-inducible promoter, $Tet^R$ | This study |
| pCIEtm::*prsA* (pCIEtm::OG1RF_10423) | pCIEtm expressing *prsA* from cCF10-inducible promoter, $Tet^R$ | This study |
| pTCV-LacSpec | Vector containing promoterless *lacZ*, $Spec^R$ | (7) |
| pTCV-LacSpec::$P_{fsrA}$ (pML200) | ~1 kb upstream of the *fsrA* gene as well as a portion of the *fsrA* gene fused to *lacZ*, $Spec^R$ | This study |
| pTCV-LacSpec::$P_{fsrB}$ (pML201) | ~500 bp that included a portion of the *fsrA* gene, the intergenic region, as well as the first 197 bp of the *fsrB* gene fused to *lacZ*, $Spec^R$ | This study |
| pTCV-LacSpec::$P_{gelE}$ (pDM7) | ~500 bp upstream of the *gelE* gene cloned upstream of *lacZ*, $Spec^R$ | This study |
| pMSP3535 | Shuttle vector with nisin-inducible promoter, $Erm^R$ | (8) |

| | | |
|---|---|---|
| pMSP3535::*gelE* (pMSP3614) | Nisin-inducible *gelE* in pMSP3535 | (9) |

$Rif^R$ = rifampicin resistance, $Fus^R$ = fusidic acid resistance, $Cm^R$ = chloramphenicol resistance, $Tet^R$ = tetracycline resistance, $Erm^R$ = erythromycin resistance, $Spec^R$ = spectinomycin resistance. Restriction enzyme sites in oligonucleotide sequences are underlined, and the enzymes are listed in the oligonucleotide names.


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